

# The ABCs of *Candida albicans* Multidrug Transporter Cdr1

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**In the light of multidrug resistance (MDR) among pathogenic microbes and cancer cells, membrane transporters have gained profound clinical significance. Chemotherapeutic failure, by far, has been attributed mainly to the robust and diverse array of these proteins, which are omnipresent in every stratum of the living world. *Candida albicans*, one of the major fungal pathogens affecting immunocompromised patients, also develops MDR during the course of chemotherapy. The pivotal membrane transporters that *C. albicans* has exploited as one of the strategies to develop MDR belongs to either the ATP binding cassette (ABC) or the major facilitator superfamily (MFS) class of proteins. The ABC transporter *Candida* drug resistance 1 protein (Cdr1p) is a major player among these transporters that enables the pathogen to outplay the battery of antifungals encountered by it. The promiscuous Cdr1 protein fulfills the quintessential need of a model to study molecular mechanisms of multidrug transporter regulation and structure-function analyses of asymmetric ABC transporters. In this review, we cover the highlights of two decades of research on Cdr1p that has provided a platform to study its structure-function relationships and regulatory circuitry for a better understanding of MDR not only in yeast but also in other organisms.**

*Candida albicans*, although a commensal in human beings, can become a cause of notorious infections in cases where the immune system is already challenged (1). Patients with AIDS and those who are undergoing chemotherapeutic modalities are always at a risk of developing *C. albicans* infections (1, 2). The advent of numerous multidrug-resistant clinical isolates of *C. albicans* has exacerbated the need for novel antifungal agents. The key mechanisms of antifungal resistance, in particular, azole resistance, include overexpression of multidrug efflux pumps, alterations in the target proteins, and adjustments in membrane sterol composition (3). The inability to accumulate detrimental concentrations of antifungal agents is attributed mainly to the ATP binding cassette (ABC) and major facilitator superfamily (MFS) classes of efflux proteins, which are overexpressed in resistant fungal isolates (3). Among the ABC transporters, Cdr1p and Cdr2p are the ones with the utmost clinical implications (4) and of the two, Cdr1p is the major determinant of azole resistance (5).

The gene encoding the Cdr1 protein (Cdr1p) was identified as a genomic DNA fragment from *C. albicans* that could functionally complement a *Saccharomyces cerevisiae* strain with *PDR5* disrupted. The resulting transformants displayed hyperresistance to several drugs (6). Since then, numerous studies carried out with this *PDR5* homologue have made several insightful contributions to our understanding of the functioning of deviant ABC pump proteins and the transcriptional networks that govern its overexpression.

This review summarizes the important findings on the structure, function, and regulation of *CDR1* and discusses the direction of future research with multidrug ABC transporters.

## THE ABC TRANSPORTOME OF *C. ALBICANS*

ABC superfamily is considered one of the largest superfamilies of proteins. These proteins contain at least one nucleotide binding domain (NBD). The NBD, which is the energy source for these proteins, further contains highly conserved motifs such as the Walker A, Walker B, and signature sequences. Most of these proteins also possess the transmembrane domains (TMDs) and are considered ABC transporter proteins. Prasad and colleagues identified

a total of 28 putative ABC protein family members in *C. albicans*, 21 of which contain TMDs (7). A protein with one NBD and one TMD is considered a half transporter. A full transporter consists of TMD and NBD in duplicate, which can be present in forward (TMD-NBD)<sub>2</sub> or reverse (NBD-TMD)<sub>2</sub> topology. These proteins are classified into six subfamilies and, according to the nomenclature adopted by the Human Genome Organization, are designated ABCB/MDR, ABCC/MRP, ABCD/ALDP, ABCF/YEF3, ABCE/RLI, and ABCG/PDR (8, 9). Genes belonging to the ABCB/MDR, ABCC/MRP, ABCD/ALDP, and ABCG/PDR subfamilies encode the transporter proteins, while ABCF/YEF3 and ABCE/RLI subfamily proteins do not contain TMDs and thus encode nontransporter ABC proteins (Table 1). A recent update of *Candida* Genome Database (CGD) assembly rearranged the open reading frames (ORFs) and deleted or merged some of the ORFs. This rearrangement reduced the total number of ABC proteins to 26, and 19 of them are ABC transporter proteins.

Of the six subfamilies of ABC transporters, ABCG/PDR is the largest, with nine members. Four members of the PDR subfamily have been characterized. Cdr1p and Cdr2p are involved in drug transport and phospholipid translocation (6, 10). Cdr3p and Cdr4p are not drug transporters but do translocate phosphoglycerides between the two monolayers of the lipid bilayer of the plasma membrane (11, 12). Thus, in *C. albicans*, only some of the members of the ABCG/PDR subfamily have been shown to be involved in clinical drug resistance. The increased expression of *CDR1* and *CDR2* in different drug-resistant clinical isolates of *C. albicans* is well documented (13). Notably, members of other sub-

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TABLE 1 ABC proteins of *C. albicans*

Subfamily <sup>a</sup>	ORF	Topology <sup>b</sup>	Size (amino acids)	Description/function	Published/suggested name	Reference
ABCG/PDR	orf19.6000		1,501	Full ABC transporter, involved in drug efflux and lipid translocation	CDR1	6
ABCG/PDR	orf19.5958		1,499	Full ABC transporter, involved in drug efflux and lipid translocation	CDR2	10
ABCG/PDR	orf19.1313		1,501	Full ABC transporter, involved in lipid translocation	CDR3	11
ABCG/PDR	orf19.5079		1,490	Full ABC transporter, involved in lipid translocation	CDR4	12
ABCG/PDR	orf19.918		1,512	Full ABC transporter, merged with orf19.919	CDR11	CGD
ABCG/PDR	orf19.5759		1,495	Full ABC transporter, similar to <i>S. cerevisiae</i> Snq2	SNQ2	CGD
ABCG/PDR	orf19.4531		1,274	ABC transporter, similar to <i>S. cerevisiae</i> YOL075C	orf19.4531	CGD
ABCG/PDR	orf19.459		1,038	ABC transporter, similar to <i>S. cerevisiae</i> Adp1	ADP1	CGD
ABCG/PDR	orf19.3120		579	Half ABC transporter, similar to <i>S. cerevisiae</i> YOL075C	orf19.3120	CGD
ABCB/MDR	orf19.1077		750	Half ABC transporter, similar to <i>S. cerevisiae</i> Atm1	ATM1	CGD
ABCB/MDR	orf19.2615		685	Half ABC transporter, similar to <i>S. cerevisiae</i> Mdl1	MDL1	CGD
ABCB/MDR	orf19.7440		1,323	Full ABC transporter, putative pheromone transporter	HST6	14
ABCB/MDR	orf19.5599		783	Half ABC transporter, similar to <i>S. cerevisiae</i> Mdl1	MDL2	CGD
ABCC/MRP	orf19.1783		1,488	Full ABC transporter, similar to <i>S. cerevisiae</i> Yor1	YOR1	73
ABCC/MRP	orf19.5100		1,606	Full vacuolar ABC transporter, involved in phosphatidylcholine import	MLT1	N. K. Khandelwal and R. Prasad, unpublished work
ABCC/MRP	orf19.6382		1,490	Full ABC transporter, similar to <i>S. cerevisiae</i> Bpt1p	BPT1	CGD
ABCC/MRP	orf19.6478		1,580	Full ABC transporter, similar to <i>S. cerevisiae</i> Ycf1 transporter	YCF1	CGD
ABCD/ALDP	orf19.7500		768	Half ABC transporter, similar to <i>S. cerevisiae</i> Pxa1	PXA1	CGD
ABCD/ALDP	orf19.5255		667	Half ABC transporter, similar to <i>S. cerevisiae</i> Pxa2	PXA2	CGD
ABCF/YEF3	orf19.2183		609	ABC nontransporter; mutation confers hypersensitivity to amphotericin B	KRE30	74
ABCF/YEF3	orf19.4152		1,050	ABC nontransporter	CEF3	CGD
ABCF/YEF3	orf19.6060		751	ABC nontransporter protein, similar to <i>S. cerevisiae</i> Gcn20	GCN20	CGD
ABCF/YEF3	orf19.7332		1,195	ABC nontransporter	ELF1	CGD
ABCE/RLI	orf19.3034		622	ABC nontransporter, similar to <i>S. cerevisiae</i> Rli1	RLI1	CGD
Other	orf19.5029		545	ABC nontransporter, similar to <i>S. cerevisiae</i> YDR061w	MODF	CGD
Other	orf19.388		320	ABC nontransporter, similar to <i>S. cerevisiae</i> YFL028c	CAF16	CGD

<sup>a</sup> ABC proteins of *C. albicans* are classified into subfamilies on the basis of nomenclature adopted by the Human Genome Organization (HUGO).

<sup>b</sup> Topology signifies the arrangement of the transmembrane and nucleotide binding domains in a forward (TMD-NBD)<sub>2</sub> or reverse fashion (NBD-TMD)<sub>2</sub>.

family are involved in a variety of functions. For example, Hst6p of the ABCB/MDR subfamily is an a-factor mating pheromone transporter (14) and the vacuolar ABC transporter Mlt1 of the ABCC/MRP subfamily is involved in *C. albicans* virulence (15).

### CLINICALLY RELEVANT Cdr1p IS A MAJOR MULTIDRUG TRANSPORTER OF *C. ALBICANS*

The pioneer study that identified Cdr1p as a major player in multidrug resistance (MDR) and implied its clinical relevance was carried out by Sanglard and his coworkers. That group used sequential isogenic isolates of *C. albicans* with increasing levels of fluconazole resistance from AIDS patients. Some of the isolates showed a remarkable 10-fold increase in the mRNA levels of then recently cloned multidrug transporter gene *CDR1* (6, 13). This report was closely followed by White's group's study, where a set of 17 sequential isolates recovered from a single HIV-infected patient who was on fluconazole therapy for 2 years was used. The levels of fluconazole resistance varied among the sequential isolates, with the 17th isolate having the highest fluconazole MIC. The *CDR1* mRNA was found to be present at constant levels in isolates 1 to 15, and its level was approximately 5-fold higher in isolates 16 and 17 (16). Upregulation of *CDR* genes was also observed in azole-resistant (AR) isolates from a marrow transplantation patient with disseminated candidiasis (17, 18). Investigation of serial *C. albicans* isolates from HIV patients undergoing fluconazole therapy for repeated oropharyngeal candidiasis episodes also showed overexpression of the *CDR1* and *CDR2* genes (19, 20).

As discussed before, the members of both the ABC and MFS superfamilies contribute to MDR in *C. albicans*; however, only *CDR1* and *CDR2* of the 19 members of the ABC family and *MDR1* of a fairly large number of MFS transporters have well-documented roles in clinical drug resistance (21, 22). A comparative study to assess the relative expression of the Cdr1 and Cdr2 proteins in a series of AR clinical isolates of *C. albicans* have revealed that Cdr1p makes a greater functional contribution than Cdr2p (23).

All of the aforementioned work and related studies helped reveal the importance of Cdr1p in clinical drug resistance, which necessitated its further characterization.

### Cdr1p: ITS INTRICATE ARCHITECTURE AND FUNCTIONING

Cdr1p is a 169.9-kDa protein built on the conventional blueprint of full ABC transporters with two homologous halves, each made up of one TMD that is preceded by an NBD. Each TMD is made up of six transmembrane helices (TMHs). The TMHs are interlinked by six extracellular loops (ECL1 to -6) and four intracellular loops (ICL1 to -4) (24) (Fig. 1A). The NBDs have the hallmark  $\beta$ -sheet subdomain containing the typical Walker A and Walker B motifs and also an  $\alpha$ -helical subdomain that consists of a conserved ABC signature sequence (21).

### THE VARIABLE TMDs ACCOMMODATE AND EXPEL A WIDE ARRAY OF XENOBIOTICS

The TMHs of fungal ABC transporters that make up the translocation pathway are highly variable in their primary sequences, unlike the well-conserved subdomains of NBDs. The understanding of drug recognition and transport by such proteins becomes ever more challenging owing to the promiscuous nature of these transporters. Cdr1p also follows the footprints of other members

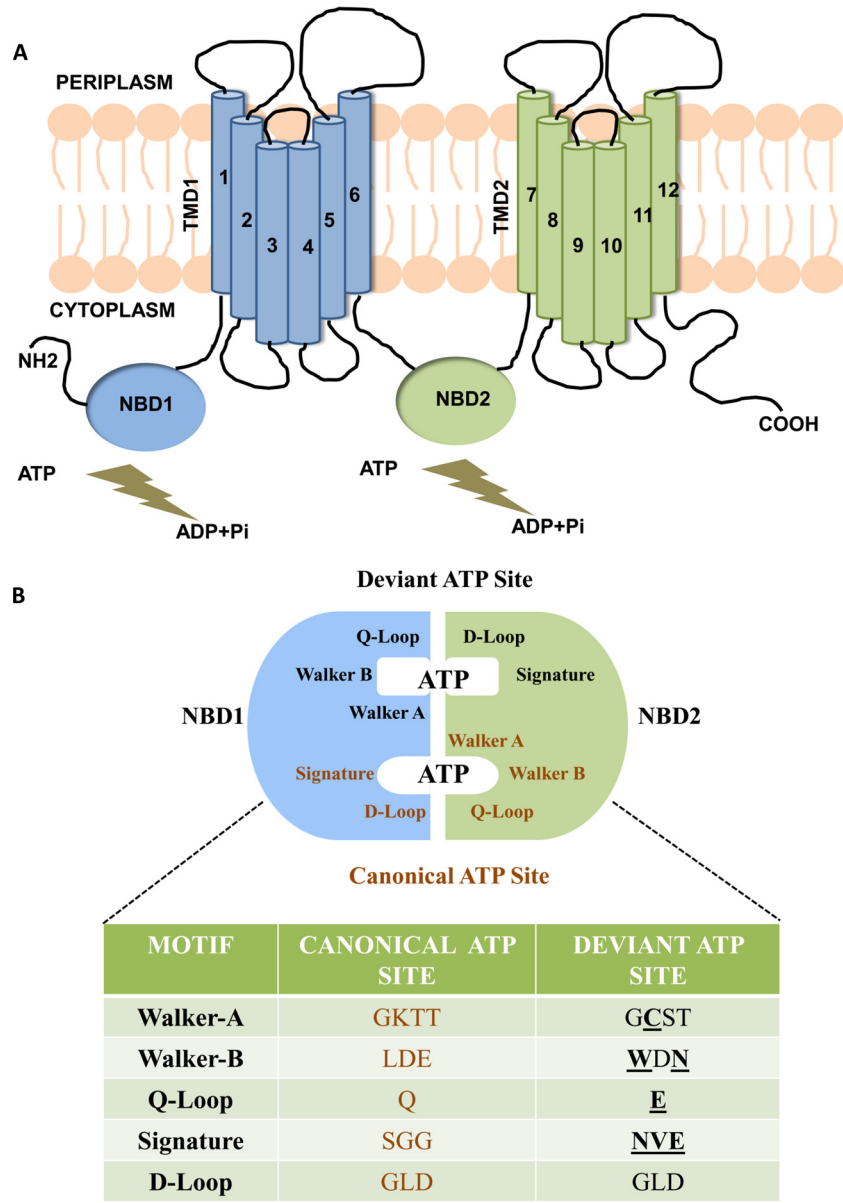
of its superfamily and has an unimaginably vast spectrum of molecules as its substrates. In order to understand the basis of the polyspecificity of Cdr1p, exhaustive computational and biochemical analyses have been undertaken by a number of research groups. Structure-activity relationship (SAR) analyses with Cdr1p predicted minimum descriptors that could distinguish between substrates and nonsubstrates of Cdr1p (25) (discussed later).

The absence of well-resolved three-dimensional (3D) crystal structural data has made it difficult to decipher the arrangement of TMHs within the drug binding site(s) of the protein. The drug binding site(s) is actually an ensemble of residues from distinct helices; hence, predicting the manner in which these residues project toward the substrate binding site and participate in substrate binding is a challenge. Furthermore, the emerging data on different ABC transporters suggest that the translocation channel within these proteins is not just straight passage but instead has many diversions that are used or sometimes even co-opted by distinct substrates. This further complicates deciphering of the structure and functioning of such proteins.

Site-directed mutagenesis is a vital tool that has been exploited to elucidate the location of a drug binding site(s) within Cdr1p. While a number of studies have revealed the importance of selected residues of helices in drug transport (26, 27), the most recent and comprehensive study to probe the drug binding site(s) was by Rawal and coworkers (28). That study involved alanine scanning mutagenesis of the entire primary structure comprising 12 TMHs. The resultant library of over 250 mutant variant proteins was overexpressed in a heterologous yeast host, and various biochemical and biophysical parameters were analyzed to gain insights into drug recognition and transport. The data from this exhaustive study revealed that within a polyspecific substrate binding site, there exist multiple overlapping minibinding sites, which probably accounts for the promiscuous nature of Cdr1p. The central drug binding cavity was found to be lined with residues from TMH1, -2, and -11 on one side and TMH4, -5, and -8 on the other. Other helices, like TMH3, TMH6, TMH7, TMH9, and TMH10, were found to contribute fewer residues to the drug binding pocket. Notably, there was no direct correlation between the importance of a residue and its degree of conservation. However, in general, the critical residues were largely hydrophobic in nature, which also coincides with the nature of the substrates subject to efflux by the protein (Fig. 2).

### THE CONSERVED NBDs POWER DRUG EFFLUX

The NBDs are an essential feature of ABC transporters. They harness the energy from ATP hydrolysis to power the expulsion of substrates across the bilayer (21). Apart from being the engine for these pump proteins, they may also play a role in substrate selection (29). One hallmark of yeast ABC transporters is that, unlike their mammalian counterparts, they elicit high basal ATPase activity that is not stimulated by the addition of substrates, indicating that transporters like Cdr1p and Pdr5p are uncoupled ABC transporters that constantly hydrolyzes ATP to ensure active substrate transport (24, 30, 31). It is believed that, by retaining high basal ATPase activity, such pumps probably remain in a transport-competent state (29). Although Cdr1p has its typical motifs (Walker A, Walker B, and ABC signature sequences) arranged in a fashion similar to that of the other representatives of its superfamily, a closer look at the amino acid sequence reveals the existence of divergent amino acids at many positions in these otherwise con-



**FIG 1** (A) Schematic representation of the predicted topology of Cdr1p. There are two TMDs and two NBDs arranged in a reverse topology. Each TMD is made up of six alpha helices. The TMHs are interlinked by four ICLs and six ECLs. The TMDs are involved in substrate binding, whereas the NBDs are responsible for ATP hydrolysis, which is essential for powering substrate efflux. (B) NBD catalytic dyad and sequence degeneracy of the residues in NBDs. The two composite ATP binding sites, *viz.*, a canonical ATP site (in orange) and a deviant ATP site (in black), are made up of contributions from both of the NBDs. The canonical ATP site is made up of Walker A and B motifs and the Q loop of NBD2 and the signature sequence and D loop of NBD1, whereas the deviant ATP site is made up of Walker A and B motifs and the Q loop of NBD1 and the signature sequence and D loop of NBD2. The atypical residues in the respective motifs are in bold and underlined.

served motifs. For instance, N-terminal NBD (N-NBD) motifs have sequence degeneracy in the Walker A (GRPGAGCST) and Walker B (IQCWDN) motifs (changes are underlined), but the ABC signature sequence (VSGGERKRVS) remains conserved. Contrary to the N-NBD, the Walker A (GASGAGKTT) and Walker B (LLFLD) motifs of the C-terminal NBD (C-NBD) are conserved, whereas the ABC signature sequence (LNNVEQRKRLT) is degenerated (21) (Fig. 1B). For this selective sequence degeneracy, there exists one canonical ATP site and one deviant ATP site. These unique substitutions were thoroughly investigated. The atypical cysteine of the Walker A motif in N-NBD (C193) has been

shown to be crucial and noninterchangeable with its equipositional counterpart (K901) in C-NBD (32, 33). A Cdr1p with either two N-NBDs or two C-NBDs is nonfunctional as well. While the chimera with two C-NBDs was not expressed properly, the chimera made up of two N-NBDs failed to localize to the plasma membrane. It was no great surprise that the variants were defective in both ATP hydrolysis and substrate efflux. Interestingly, the variant with two N-NBDs could be rescued to the plasma membrane when cells expressing the variant were exposed to drug substrates. It is noteworthy that although the rescued N-NBD variant was defective in ATP hydrolysis and

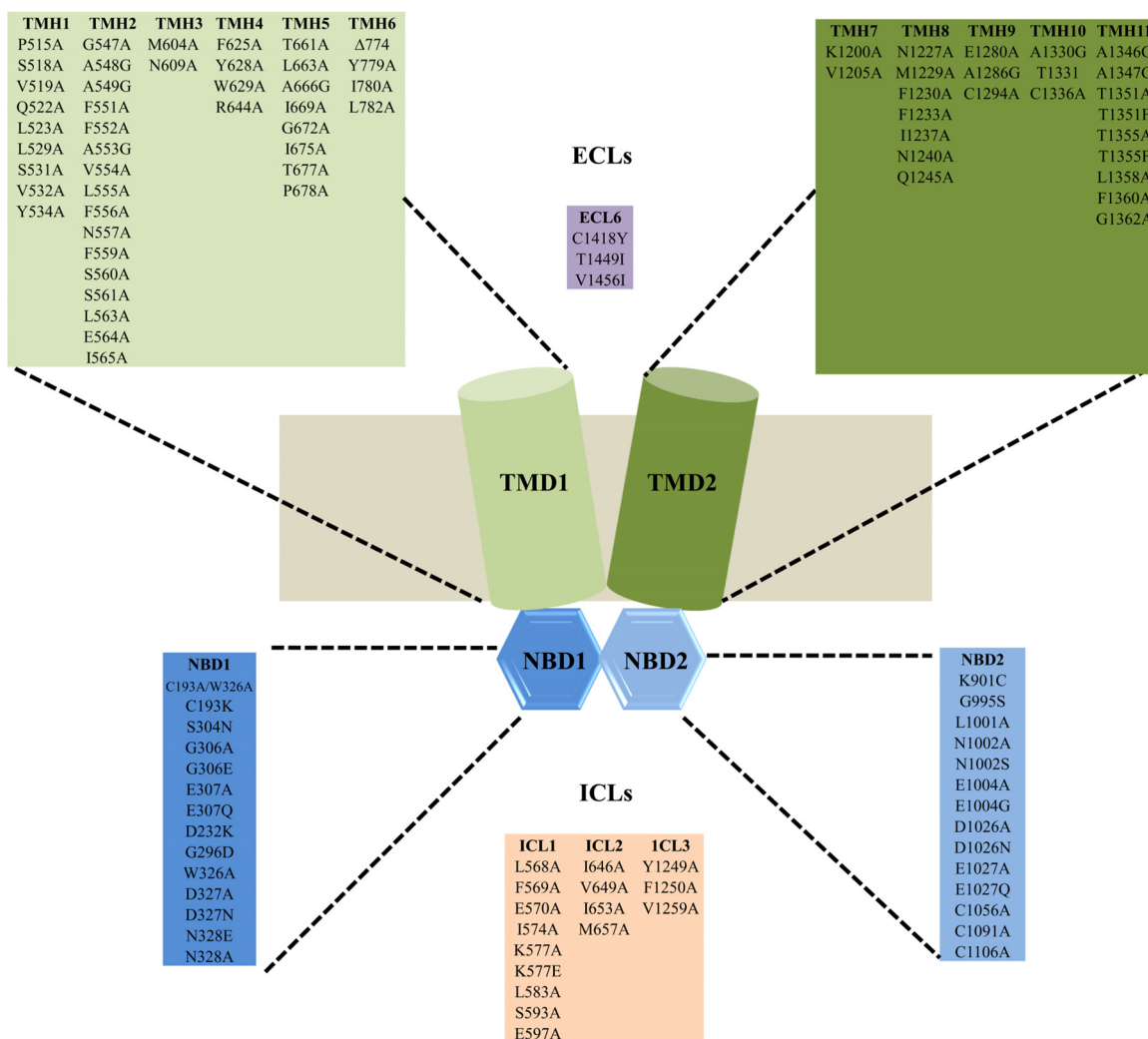


FIG 2 Residues of TMDs, NBDs, ECLs, and ICLs of Cdr1p whose replacement enhances susceptibility to drugs. The data are compiled from several publications (24, 26–28, 33, 36–38, 43, 45).

substrate transport, substrate binding *per se* was not affected. These observations highlighted the positional significance of the two NBDs (34).

The sequence degeneracy of N-NBD in fungal ABC transporters led to the assumption that probably only C-NBD is the main hub of ATP catalysis, which is required for resetting the TMDs, whereas N-NBD's role may be architectural, allowing interaction with the opposing NBD while in its nucleotide-bound form, or it could play a regulatory role as well, similar to that of the N-NBD of the human ABC transporter cystic fibrosis transmembrane conductance regulator (29). The experiments that followed to investigate the functional relevance of sequence degeneracy in isolated domains revealed that atypical C193 of Walker A and W326 of Walker B of N-NBD are in close proximity in the ATP binding pocket, where the former residue participates in hydrolysis while the latter impacts the binding of the nucleotide (35, 36). Interestingly, well-conserved D327, which is otherwise the catalytic carboxylate in other ABC transporters, appears to have a new role and behaves as a catalytic base involved in ATP hydrolysis in this transporter (37). Be-

sides the conserved signature sequence residues of N-NBD, the role of divergent signature sequence residues of C-NBD has also been assessed. It was no great surprise that the two conserved signature residues S304 and G306 were found to be important for ATP catalysis. Interestingly, their equipositional counterparts in C-NBD, *viz.*, N1002 and E1004, turned out to be functionally essential too. While an N1002A mutant was hypersusceptible to all of the drugs tested, with impaired R6G efflux and ATPase activity, an E1004A mutant showed selective susceptibility to certain drugs, with marginally reduced R6G efflux and wild-type ATPase activity (38). These striking observations provided further instances of an emerging trait of NBD residues, influencing substrate specificity.

Taking these results together, it could be said that the divergent residues have assumed distinct roles during the course of evolution and have their own unique importance. One obvious conclusion that comes out of such atypical substitutions is that there might be some distinct mechanism operating at the level of the NBDs that is peculiar to Cdr1p. Further study of Cdr1p

and other fungal ABC transporters could help solve this enigma.

### EXTRACELLULAR LOOPS: THE ADJUSTABLE LIDS

The random mutagenesis of Pdr5 has highlighted the importance of ECL residues in protein assembly, cell surface localization, and substrate specificity (39). In one particular study with Cdr1p, cysteines from ECL6 were found to be critical for drug resistance (26). The study by Niimi and coworkers with RC21v3, a surface-active D-octapeptide inhibitor of Cdr1p, showed that it could chemosensitize cells expressing Cdr1p to fluconazole. The spontaneously recovered chromosomal suppressors localized within or close to the ECLs and could alleviate RC21v3 inhibition. Interestingly, most of the suppressor mutations introduced positive charges. Since RC21v3 is a cationic-substrate-active inhibitor, there is a possibility that the positive charges introduced as suppressors might actually repel the peptide. The data suggested that the peptide might interact with the extracellular surface of Cdr1p, blocking conformational changes and locking the protein in a closed state. This would result in inaccessibility to the substrate binding sites. It is also possible that the inhibitor can physically block substrate transport by occupying the egress channel at the surface of the transporter, which the substrates otherwise enter before being expelled out of the bilayer (40). Although it is mere speculation, the emerging consensus regarding the ECLs is that they act like lids/gates and can alter substrate specificity and transport, a possibility worth examining in the future.

### INTRACELLULAR LOOPS: THE SIGNALING INTERFACES

In contrast to the ECLs, the ICLs are much more conserved in their primary sequence but are shorter (31). Structure-function studies with yeast ICLs suggest that these loops actually serve as transmission interfaces between the TMDs and NBDs (41, 42). A recent attempt to deduce the roles of intracellular-loop residues involved the replacement of each of the 85 ICL residues with a single alanine. The results revealed that close to 18% of the ICL residues, when replaced with alanine, yielded mutant protein variants that, upon expression, displayed enhanced drug susceptibility in host cells. Of the susceptible mutants, most displayed uncoupling between ATP hydrolysis and drug transport. Chromosomal suppressors for two such ICL1 mutants (I574A and S593A) fell near the Q loop of C-NBD (R935T) and in the Walker A motif (G190R) of N-NBD, respectively. Instead of directly communicating with the mutants, the suppressors actually functioned by restoring the coupling interfaces and reestablishing essential contacts between the NBD and TMD, which were otherwise distorted upon the mutation of the two aforementioned ICL1 residues (43). Another study by the same group identified an ion pair between K577 of ICL1 and E315 of N-NBD that was critical for proper folding and localization of the protein (44). That study pointed toward a new role for NBD/ICL interacting residues in protein folding/trafficking. Taken together, all of these reports indicate that the ICL-NBD junctions play crucial roles and are essential for the protein's structure and function.

### Cdr1p IS A PROMISCUOUS TRANSPORTER

The hallmark of all ABC transporters is their promiscuous nature. Cdr1p also follows the legacy and has a vast spectrum of structurally unrelated molecules as its substrates (Fig. 3). Determining

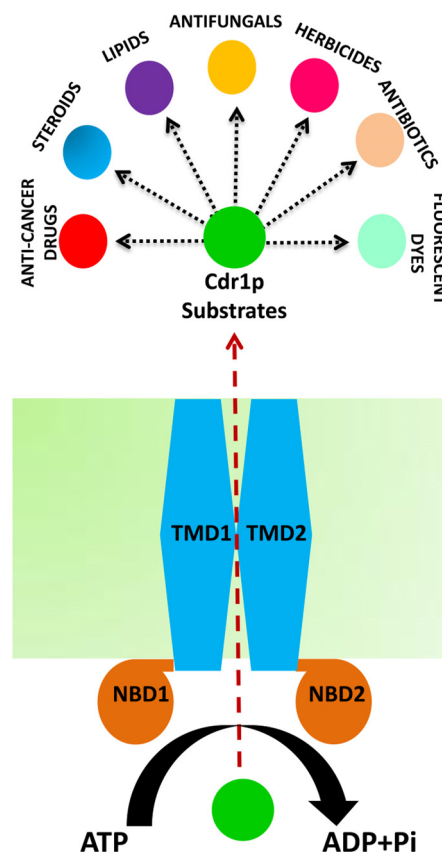


FIG 3 Schematic representation of the promiscuous nature of Cdr1p.

how Cdr1p recognizes such a diverse nature of substrates, which includes xenobiotics, drugs, lipids, etc., remains a challenge. The worrisome situation of MDR has been demanding a better understanding of the basis of the promiscuity of Cdr1p and similar proteins. SAR analysis has been extensively employed to evaluate the polyspecificity of such proteins. It has been proposed that Cdr1p substrates generally possess a high hydrophobicity index. In addition, molecular branching, high aromaticity, and the presence of an atom-centered fragment (R-CH-R) are some of the other features that are essential for the substrates of Cdr1p (25). Notably, Cdr1p has a large number of aromatic residues in the binding pocket and has significant clustering of aromatic residues near the ectodomains. Thus, substrates with high aromaticity could be involved in stacking interactions with such residues. Since the binding cavity is predicted to be large (28) and numerous residues are involved in its interactions with the substrates, it is likely that branching increases the reactive surface area for substrate molecules, which results in better passage through the channel.

### Cdr1p TRANSPORTS UNCONVENTIONAL MOLECULES

As discussed above, Cdr1p has the ability to extrude a plethora of structurally unrelated substrates. These include different classes of antifungals, fluorescent dyes, plant products, herbicides, anticancer drugs, and many more (Fig. 3). Apart from these, the protein also transports physiological substrates like steroids and phosphoglycerides. Human steroid hormones such as  $\beta$ -estradiol and corticosteroids are actively expelled by Cdr1p. Interestingly, while

Cdr1p could manage  $\beta$ -estradiol and corticosterone efflux, it is unable to recognize and manage the efflux of another closely related steroid hormone, progesterone. Steroid transport could be competed by an excess of drug substrates, implying that Cdr1p has binding sites in common with the drugs (45). Since other ABC transporters can also transport steroids, it is reasonable to assume that these hormones could also be physiological substrates for these transporters (21).

Steroids have definitive implications in *Candida* infections. For instance, vulvovaginal candidiasis is a common ailment; an elevated level of estrogen and high glycogen content in vaginal secretions during pregnancy predispose women to the disease (46). Furthermore, 17- $\beta$ -estradiol could positively influence the germination of *C. albicans* (47, 48). Interestingly, it was shown that the 17- $\beta$ -estradiol effect was brought about in a dose-dependent, as well as strain-dependent, manner (48). Since the hyphal form is responsible for tissue invasion, these reports imply that steroid hormones could act as cues for the virulence of *C. albicans*. The steroid signaling pathways are largely unknown in *Candida*, though steroid binding proteins exist in *C. albicans* (49). It was also observed that brief exposure of *Candida* cells to steroid hormones results in upregulation of the *CDR1* gene (50). In this scenario, it is plausible that there is an as-yet-uncharacterized link between MDR and the steroid response pathway.

Asymmetric distribution of phospholipids across plasma membrane is quite well known (51). Perturbations in lipid asymmetry have profound clinical consequences (51, 52). Cdr1p, which itself prefers to be localized within microdomains enriched with sphingolipids and ergosterol, is also responsible for the asymmetric distribution of phosphoglycerides in *C. albicans* plasma membranes (53, 54). The floppase activity of Cdr1p has also been shown with purified and functionally reconstituted protein (55). Similar to steroid transport, phosphoglyceride translocation could be outcompeted by certain drugs, which again not only points to the polyspecificity of the transporter but also highlights the overlapping binding sites within the large, flexible drug binding cavity. Together, these findings imply that, during the course of evolution, *C. albicans* and other fungi have learned to utilize their available repertoire of transporters to bring about the efflux of xenobiotics, albeit they have their own set of physiological substrates.

### MODULATORS/INHIBITORS OF Cdr1p

Among the different strategies employed to overcome MDR, inhibition of the drug extrusion activity of MDR pumps is one principal approach. Similar to yeast cells, MDR is an obstacle to effective chemotherapy in cancer cells, where ABC transporters play an important role. Many clinically relevant anticancer drugs have been identified that function as modulators of human ABC transporters (56). Although research on modulators of yeast MDR pump proteins is still in its infancy, there are certain compounds, such as enniatins, milbemycins, isonitrile, and unnarmicins, that have been demonstrated to modulate drug efflux by inhibiting the fungal multidrug transporters (23). Niimi and coworkers have identified the D-octapeptide derivative RC21v3 as a potent inhibitor of Cdr1p (40). We have shown earlier that a disulfiram drug (Antabuse) inhibits the oligomycin-sensitive ATPase activity of Cdr1p (57). In addition, the polyphenol curcumin and the quorum-sensing molecule farnesol act as modulators of Cdr1p (58, 59). Notably, disulfiram, curcumin, and farnesol also potentiate

antifungal activity and thus display synergy with certain antifungals. Recently, it was shown that the peptide mimic of TMH8 of Cdr1p could act as inhibitor of efflux activity and also synergize with fluconazole under both *in vitro* and *in vivo* conditions (60). Despite insufficient information, the use of modulators and peptide mimics as inhibitors of efflux pumps represents a promising strategy for successful antifungal therapy.

### REGULATION OF *CDR1* TRANSCRIPTION

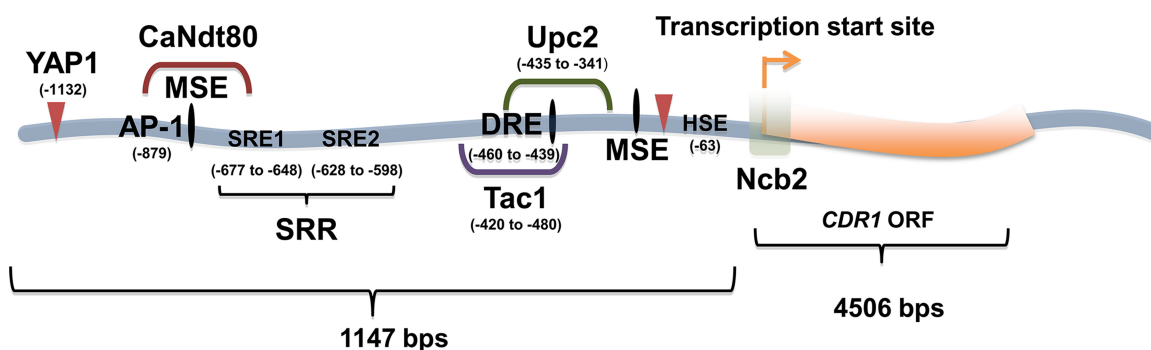
**(i) Transcription factors: the hidden players.** Coinciding with the role of *CDR1* as a major multidrug transporter in clinical antifungal resistance, it is a well-regulated gene. The transcription of *CDR1* is controlled by several well-characterized *trans* factors that have been shown to interact with a host of *cis* elements interspersed in its promoter (discussed later). Tac1 is a prominent transcription regulator of *CDR1* and is often associated with gain-of-function mutations resulting in hyperresistance in clinical *Candida* isolates. Genome-wide transcription profiling and its comparison in matched pairs of AR and azole-susceptible (AS) clinical isolates revealed the hyperexpression of *CDR1*, *CDR2*, and 12 other genes. Interestingly, this effect was diminished in *tac1Δ/Δ* mutant AR strains. Notably, chromatin immunoprecipitation (ChIP)-on-chip assays validated the binding of *TAC1* to the promoters of *CDR1* and *CDR2* (61).

Genome-wide location analysis of Upc2, a zinc cluster family transcription factor that is implicated in sterol biosynthesis, revealed *CDR1* as one of its target genes, thus implying its importance in MDR. As expected, the *upc2Δ/Δ* mutation results in azole hypersusceptibility. Notably, Upc2 acts as an activator or repressor, depending on its own activation. For example, *UPC2* is activated by lovastatin or hypoxia and *CDR1* expression was down-regulated during lovastatin treatment and upregulated in the absence of *UPC2*. Interestingly, hypoxia does not have the same effect as *UPC2* upon *CDR1*, implying a complex mechanism of regulation (62).

*CAP1*, a bZIP transcription factor, came to light as a functional homologue of *YAP1* in *S. cerevisiae* that confers fluconazole, cycloheximide, and 4-nitroquinoline *N*-oxide resistance and, upon overexpression, also governs *CDR1* expression (63, 64). Genome-wide occupancy and expression profiling of *CAP1* revealed that it binds to the promoters of a host of genes involved in the oxidative stress response and drug resistance, including *CDR1*, phospholipid transport, etc. Strikingly, not only the transcription factors that are implicated in drug resistance and are global regulators control *CDR1* expression but those that play roles in cell division and proliferation also impact its transcription. For instance, the overexpression of CaNdt80, a functional homolog of meiosis-specific transcription factor Ndt80, in *S. cerevisiae* induced the transcription of *CDR1*, which could be prevented by creating a mutation in its activation domain (65, 66).

Recently, functional analysis of transcription factors was undertaken by employing artificial activation. This involves fusion of the Gal4 activation domain to the C terminus of the full-length protein. This strategy has helped in the characterization of the role of Mrr2 in fluconazole resistance mediated through overexpression of *CDR1* (67).

**(ii) Promoter analyses: the governing authority.** *In silico* analysis revealed that the *CDR1* promoter houses a host of regulatory elements, including, AP-1, yeast AP-1 (*YAP-1*), heat shock elements (HSEs), and MDRNF1 (or drug regulatory elements) (68)



**FIG 4** *cis* regulatory elements in the *CDR1* promoter. The model shows the approximate locations of *cis* elements. ORF size is condensed to emphasize the promoter. The DRE spans positions  $-460$  to  $439$  (69); the Tac1 binding region spans positions  $-420$  to  $-480$  (61); Upc2 is located roughly between positions  $-435$  and  $-341$  (62); the SRR consists of two distinct regions, SRE1 at positions  $-677$  to  $-648$  and SRE2 at positions  $-628$  to  $-598$  (49); mammalian AP-1 is present at position  $-879$ ; an HSE is located at position  $-63$  (68); the *CDR1* promoter houses three mid-sporulation elements (MSEs) to which CaNdt80 binds at positions  $-270$ ,  $-438$ , and  $-835$  (65); two YAP-1 elements are present at positions  $-1132$  and  $-161$  (68); and Ncb2 has been shown to bind near the transcription start site of *CDR1* in AR isolates (72).

(Fig. 4). Apparently, mutational analysis demonstrated that most of the regulatory elements are positioned in the proximal promoter region, while the one required for response to miconazole stress, AP-1, is situated at the distal promoter (68). Since human steroid hormones induce *CDR1* expression, its promoter was examined for the presence of steroid-responsive elements. The search resulted in the identification of a steroid-responsive region (SRR) comprising two distinct *cis* elements, SRE1 and SRE2, responsive to progesterone and progesterone and  $\beta$ -estradiol, respectively (49). Transcriptional analysis of *Candida* cells upon exposure to estrogen show the upregulation of *CDR1* and *CDR2*, indicating a plausible connection with drug resistance (48). Response to estradiol subsequently assisted in the delineation of basal expression elements and drug-responsive element I (DRE<sub>I</sub>), which were specific to induction by estradiol. Regulation of *CDR1* by a number of unrelated transcription factors and the presence of many *cis* regulatory elements in its promoter suggest that molecular networks leading to its activation under a particular condition dynamically and delicately control the expression of *CDR1*. Although both the *CDR1* and *CDR2* promoters contain DREs, the basal expression of *CDR2* is much lower than that of *CDR1*, suggesting an intricate regulation pattern for *CDR1* (69).

Increased *CDR1* transcription and mRNA stability were two predominant factors implicated in the development of azole resistance in AR isolates in comparison with matched AS isolates (70). In another study, it was found that poly(A) polymerase homozygosity and hyperadenylation are responsible for the increased stability of *CDR1* mRNA (71). However, the half-life of Cdr1p did not change between AS and AR isolates, thus ruling out any possible role for Cdr1 protein stability in drug resistance (70). Recently, the transcriptional regulation of *CDR1* by Ncb2, the  $\beta$  subunit of the NC2 complex, a heterodimeric regulator of transcription, has been reported (72). ChIP-on-chip analysis revealed that the global regulator Ncb2 could bind to the *CDR1* promoter in both AR and AS isolates; however, the preferential recruitment of Ncb2 to the TATA box region of *CDR1* under activation (AR) and a shift at the TATA upstream region under repression (AS) were noticed. That study suggested that the specific enrichment of Ncb2 in AR isolates leads to transcriptional activation, whereas higher occupancy and positional rearrangement lead to repression, of *CDR1* (72).

## FUTURE DIRECTIONS

To elucidate the mechanistic details of drug recognition and transport by Cdr1p, exhaustive biochemical studies have been undertaken by us and a number of other groups. Despite such valiant efforts, complete understanding of the functioning of Cdr1p and similar medically important ABC transporters remains elusive, mainly because of the unavailability of 3D structural data. With the advent of advanced methodologies to determine the structures of membrane proteins, it seems this drought will end in the near future. The importance of asymmetry in the NBDs, arrangements of the TMHs within Cdr1p, substrate promiscuity, and the sequence of events in the catalytic process of the protein could be understood only with an appropriate blend of biochemistry and structural biology. Conjointly, high-throughput genome-wide analyses should be employed to dissect novel proximal and distal factors controlling the temporally varying on-off switch for *CDR1* expression, which has crucial implications in azole resistance.

In a nutshell, novel therapeutic agents are the need of the hour to circumvent the menace of MDR. Such therapeutics should not be limited to pump inhibitors but should also include molecules targeting Cdr1p transcription, translation, and plasma membrane sorting.

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